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Analysis of Vaname Shrimp DNA Fragment Resistant to White Spot Virus Syndrome

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ABSTRACT

The attack of WSSV in vannamei shrimp cultivation is still common. Shrimp quality improvement can be made through selection with the help of markers (marker-assisted choice). This study aimed to evaluate the DNA fragment profile of white shrimp that was resistant to WSSV disease. The analysis was performed using the PCR-RAPD method. WSSV challenged four groups of 100 vannamei shrimp, then DNA was extracted from live and dead shrimp. The results showed that 2 of the 17 primers tested had high potential as markers, namely OP-09 and OPD-2. PCR products with OPC-09 primers had specific DNA bands measuring about 1.2 kb in all post-challenge WSSV resistant shrimp individuals. The amplification results using OPD-02 primers showed a particular band of DNA with a length of about 1.0 kb, with 60% of the appearance in WSSV-resistant shrimp. In contrast, the WSSV-susceptible shrimp group did not have specific DNA fragments. Thus, the two RAPD primers had a high chance of being used in the selection with the help of markers to produce WSSV resistant shrimp.

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1. Introduction

White spot syndrome virus (WSSV) is a pathogenic shrimp virus that causes considerable losses to shrimp farmers worldwide. In recent years, shrimp production has decreased sharply as a result of WSSV disease. Infected shrimp shows white spots on the carapace and body, leading to a

high mortality scale of 80-100% within days of infection (Chou et al., 1995; Nakano et al., 1994). Disease expansion control is carried out by improving the quality of shrimp seeds, significantly increasing disease resistance. One disease prevention method is through immunostimulants to increase shrimp's natural immunity against disease and enhance shrimp's genetic performance through cross-breeding and selective breeding. Indonesian shrimp culture is still constrained by limited in shrimp quality, quantity, and continuity. The need for good seeds is essential for shrimp pond cultivation in the community, directly affecting national shrimp production. However, superior fish seeds can only be produced from genetically superior broodstocks besides the influence of water and feed quality in their maintenance (Migaud et al., 2013; Nyonje et al., 2018).

The program to increase vannamei shrimp production begins with efforts to prepare prospective vannamei shrimp through the rigorous selection to obtain vanname shrimp broodstock with advantages, including disease resistance (Trang et al., 2019). Vanname shrimp breeding efforts have an excellent opportunity to provide high genetic diversity through cross-breeding and selective breeding, which are believed to increase the breeding value (Nguyen, 2016). Superior shrimp broodstock will reduce their offspring's superiority so that the next generation will have more value (Browdy, 1998). The results of the best selection of Vanname broodstock were then analyzed for the Vanname DNA profile using the Random Amplified Polymorphism DNA (RAPD) method (de Freitas et al., 2005). The basic principle of the RAPD method is to utilize one synthetic oligonucleotide as a non-specific primer to amplify a small number of DNA template samples under conditions of relatively low PCR annealing temperature (Law et al., 2015). The purpose of this study was to study the DNA fragment profile of vaname shrimp which has superior properties of WSSV disease resistance.

2. Material and methods

2.1 Vaname shrimp preparation

The selection results obtained four groups with A, B, C, D codes. Furthermore, 100 Vannamei with the best phenotypic and genotypic characteristics were adapted for one week in a 2-ton capacity fiber tub equipped with aeration equipment. Moreover, the commercial shrimp pellets were used as much as 5% of the biomass conducted every 4 hours.

2.2 WSSV antigen preparation

The WSSV virus used for the challenge test in this study was a local isolate provided by the Laboratory of the Center for Brackish Water Cultivation, Situbondo. For the cohabitation challenge test, the number of copies of the WSSV virus in shrimp muscle was quantified by the Real-Time PCR method using a primer, Forward 5'-TGGTCCCGTCCTCATCTCAG-'3, Reverse 5'-GCTGCCTTGCCGGAAATTA-'3, and 5'/56 FAM/AGCCATGAAGAATGCCGTCTA-'3 Probe to determine the number of virus copies infected shrimp.

2.3 Challenge Test Procedure

This test used four groups containing 100 vannamei, each group with an average weight of 1.5 g. The test for WSSV transmission was carried out through cohabitation, in which the shrimp were fed with shrimp meat containing WSSV, 5% of the shrimp biomass. Observation of WSSV infection was carried out every day during the challenge test.

2.4 Genomic DNA extraction

Live and dead shrimp in each group, after the challenge test with WSSV, were taken their pleopod for genomic DNA extraction. Genomic DNA extraction using a kit (Roche, Mannheim, Germany) according to the procedure. DNA concentration was measured using Nanodrop (IMPLENED, NanoPhotometerTM Pearl, Germany). Furthermore, the genomic DNA was stored at -20 ºC until it was used for the PCR process.

Shrimp genomic DNA results were detected β-actin shrimp using Forward 5'-CCTCCACCATGAAGA-'3 and Reverse 5'-CACTTCCTGTGAACAATTGATGGTC-'3 primers. The amplification process was carried out using the following reaction compositions: 5x reaction buffer, 10 mM dNTP; 25 mM MgCl2, 2.5 UTaq DNA polymerase (Promega, USA), 50 µg / ml DNA template, and one pmol primer with a total volume of 10 µl. The amplification cycle used one denaturation cycle at 95 ºC for 5 min. Then 40 multiplication cycles consisting of 95 ºC for 30 s, 62.9 ºC for 30 s, and 72 ºC for 30 s, and one final cycle at 72 ºC for 5 min.

2.5 PCR-RAPD analysis

In the initial stage, 17 types of primers were tested in the PCR process to determine which primers could be used to amplify Vanname shrimp DNA. Furthermore, 8 RAPD primers were used in the PCR process to amplify WSSV resistant Vanname shrimp's DNA. The eight RAPD primers include OPA-11, OPA-17, OPB-06, OPC-09, OPD-02, UBC-456, UBC-457 and YNZ-22 primers. Two primers were selected in the final stage, which produced varying length and visible DNA bands, namely OPC-09 and OPD-02.

The amplification process was carried out using the Polymerize Chain Reaction (PCR) method. The reaction composition consisted of 5x reaction buffer, 10 mM dNTP; 25 mM MgCl2, 2.5 UTaq DNA polymerase (Promega, USA), 50 ng DNA template, and 1 pmol primer with a total volume of 10 µl. The PCR cycle used in stage I amplification was 45 cycles at 92oC for 1 minute, 35oC for 1 minute 30 seconds, and 72 ºC for 1 minute. Furthermore, stage 2 is the last cycle at 4oC for infinity.

The electrophoresis amplification results on agarose gel with a concentration of 2% used a voltage of 70 mV for 2 hours in a 1X TAE buffer solution. DNA was visualized with 3x Red Gel, then observed under UV light and documented with a Geldoc Camera.

2.6 Water quality

Water quality parameters measured included salinity, pH, ammonia, and dissolved oxygen. Measurements were made before and after the challenge test.

2.7 Data analysis

The DNA banding pattern of the RAPD results was then translated based on the presence or absence of the bands. The data is used in determining the RAPD band pattern as polymorphic or monomorphic. According to Jorde et al (1995), DNA bands are categorized as polymorphic if the resulting fragments do not appear in some samples. If all bands appear (monomorphic) in all populations were classified as specific markers. Furthermore, DNA fragments were analyzed descriptively by adding up each DNA band from PCR amplification and calculating the percentage of polymorphism samples from each living (H) and susceptible (M) group after the challenge test WSSV.

3. Results and Discussion

3.1 WSSV Challenge Test

Exposure to WSSV with 466,783 copies of the WSSV virus per shrimp group showed clinical symptoms that appeared in the tested shrimp, including decreased feed appetite and weakness. The mortality of shrimp susceptible to WSSV occurred partially for one week in a row and occurred in each family of Vanname shrimp. The number of copies of virus isolates is shown in the following table:

Table 1. WSSV isolate used in the Vanname shrimp challenge test

No	Name	Average of CT	*copy* in average
1	Standar 10^3	28.651	1 000
2	Standar 10^5	22.054	100 000
3	Standar 10^7	15.477	10 000 000
4	Standar 10^8	12.588	1 000 000 000
5	isolate	27.655	466.783
6	Vannamei resistant to WSSV A	30.64	67 507.19
7	Vannamei resistant to WSSV B	28.55	16 945.90
8	Vannamei resistant to WSSV C	25.16	196 673.16
9	Infected Vannamei A	18.19	28 670 788.00
10	Infected Vannamei B	20.65	4 903 998.00
11	Infected Vannamei C	19.56	10 701 968.00
12	Infected Vannamei D	19.48	11 348 825.00
13	Negative control	Undetermined	

Three groups of vaname shrimp with codes A, B, and C were resistant to WSSV disease from the challenge test.

3.2 DNA fragment analysis

The RAPD analysis phase begins with detecting β-actin Vanname shrimp from 4 different resistant groups (Life) and susceptible (Dead) after the challenge test. The amplification results showed the uniformity of the DNA bands in both live Vanname shrimp (columns 1–4) and dead (columns 5–8), namely at 150 bp, in Figure 1. This shows that the DNA extraction and reaction composition of the Vanname shrimp PCR analysis on all treatments followed the procedure and produced a clear DNA band (Triana 2010).

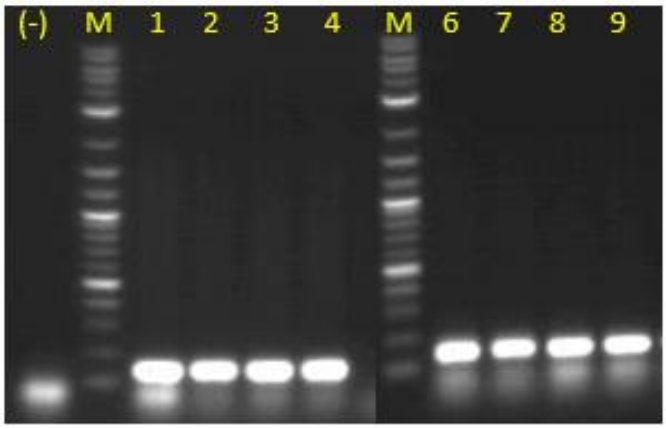

Figure 1. Electrophoresis results of β-actin Vanname from 4 groups (A, B, C, D) that were resistant (Life) and susceptible (Dead) after the challenge test. M: Marker 0.1-10 kilobase (kb), lane (1-4) β-actin Vannamei live after WSSV challenge test from groups A, B, C, D, lane (5-8) β-actin Vannamei susceptible to WSSV post-challenge test from groups A, B, C, D.

In the initial stage, DNA screening of Vanname shrimp was carried out using 17 random primers that had not been previously known, which could be used as genetic markers for RAPD in Vannamei shrimp. The RAPD marker is a modified PCR technology technique to amplify the genome using "arbitrary primary" or random primers (Welsh et al 1990; Williams et al 1990).

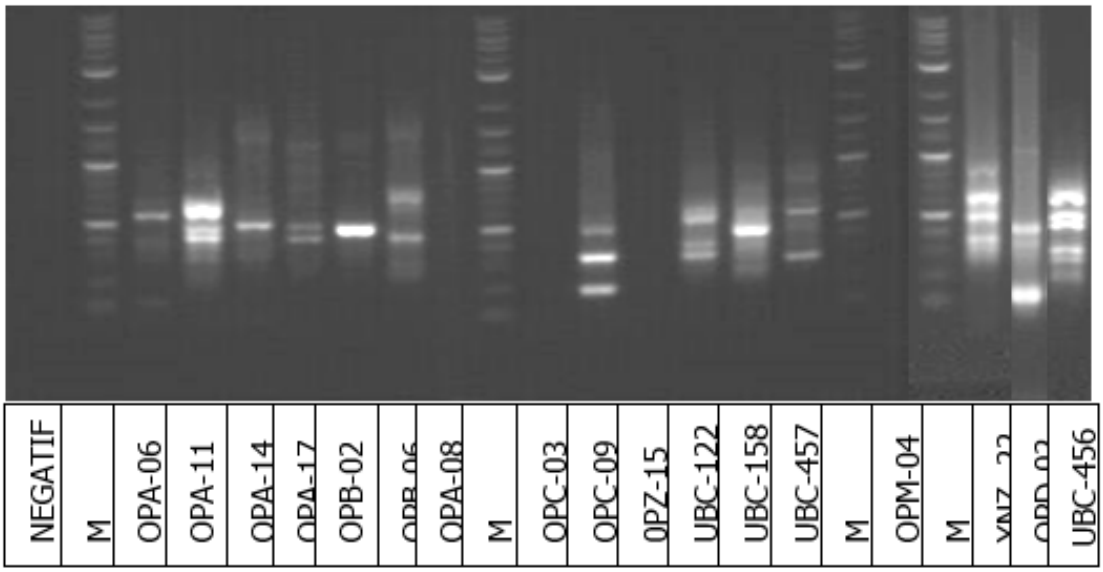

Figure 2. The DNA electrophoresis results of PCR-RAPD using 17 different primers; M: Marker 0.1-10 kilobase (kb).

Figure 2 shows the results that not all 17 primers can attach to the genomic DNA. Primers OPA-08, OPC-03, OPZ-15, and OPM-04 in the PCR process did not produce DNA bands. This is because the primers and the template DNA are not complementary. If the primers and the printed DNA are complementary, the primer will stick to different sites on the two strands of printed DNA. PCR products in DNA bands will be obtained if the primary attachment site is within an amplifiable distance (Mamiatis et al 1985; Tingey et al 1992). The number of DNA bands produced by each primer depends on the distribution of homologous sites with primary sequences in the genome (Mamiatis et al 1985).

In the screening stage of WSSV resistant Vannamei molecular markers, eight primers were used, which produced more than one different DNA band. The primers were OPA-17, OPB-06, OPC-09, OPD-02, 456, 457 and YNZ-22. Following Matondang et al (2001) statement, the RAPD method requires random primary selection, which gives polymorphism results; namely, the RAPD bands produced more than one band.

Because primers OPA-11 and UBC-122 produced coincided DNA bands, only one of the primers with a more evident DNA band would be used, and the OPA-11 primer was more likely to be used.

Table 2. Primary DNA sequences that produce DNA banding patterns.

Primer	DNA Sequence	References	Band	Line position
OPA-06	GGTCCCTGAC	(Hizer et al 2002)	1	3
OPA-11	CAATCGCCGT		5	4
OPA-14	TCTGTGCTGG	(Khetpu 2005)	1	5
OPA-17	GACCGCTTGT	(Hizer et al 2002)	3	6
OPB-02	TGATCCCTGG		1	7
OPB-06	TGCTCTGCCC		3	8
OPC-09	CTCACCGTCC		3	12
OPD-02	GGACCCAACC	(Hizer et al 2002)	4	21
UBC-122	GTAGACGAGC	(Khetpu 2005)	4	14
UBC-158	TAGCCGTGGC	(Khetpu 2005)	1	15
UBC-456	GCGGAGGTCC	(Klinbunga et al 2000)	5	22
UBC-457	CGACGCCCTG	Diaz et al 2007	3	16
YNZ-22	CTCTGGGTGTCGTGC	(Klinbunga et al 2000)	4	20

In the next PCR process for analysis of samples of 3 groups of WSSV resistant vanname, OPC-09 and OPD-02 primers were used because these primers produced a band pattern that had varied and precise lengths. The electrophoresis results of PCR products using OPC-09 and OPD-02 primers for all samples are shown in Figures 3 and 4, while the number of DNA fragments produced by each sample could be seen in Table 2.

The amplification results using the OPC-09 primer showed that the specific marker bands of Vanname family A, B, and C shrimp were two ribbons with a 200 and 500 bp, in Figure 3.Both DNA bands were owned by all shrimp, both live shrimp before infection. WSSV (control) and resistant shrimp and died after WSSV infection.

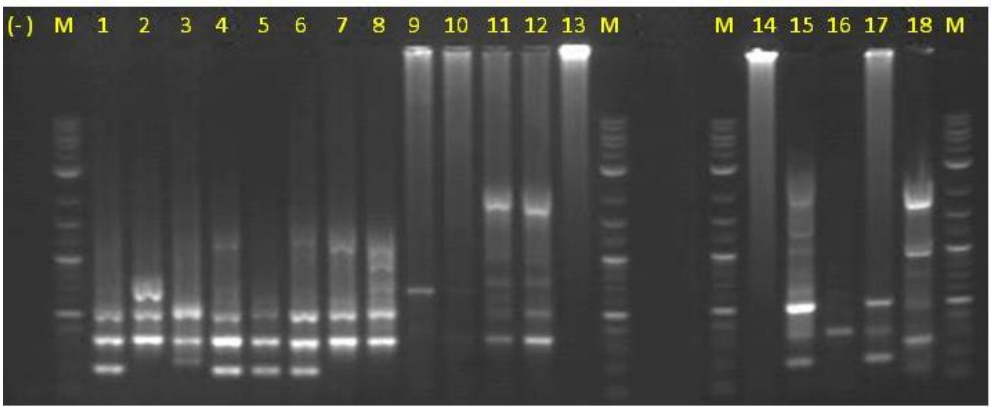

Figure 3. Results of DNA electrophoresis of resistant/live and susceptible/dead Vanname shrimp PCR-RAPD products after the WSSV challenge test. The PCR process uses the OPC-09 primer. M: Marker 0.1-10 kilobase (kb), (-) negative control, lane (1-4) without WSSV infection in group A,

B, C, D, lane (5-8) live WSSV infection in group A, B, C, D, lane (9-13) Vanname Nusantara I test results LD50 WSSV, lane (14-17) WSSV infection died in groups A, B, C, D, lane (18) isolates WSSV (10^{-1}) LD50.

The DNA banding pattern of the control Vanname shrimp group and those that were resistant (life) WSSV was generally different from those that were susceptible (dead) after the challenge test. All isolates from live shrimp before and after the challenge test showed specific DNA bands of 200 and 500 bp markers, but particular live shrimp markers were resistant to WSSV at 1650 bp. The 1650 bp DNA band did not appear at all in susceptible (dead) shrimp. The average number of DNA bands that were alive in three fragments with varied patterns was greater than that of Vanname shrimp that died 1-2 segments. It indicates that the use of the OPC-09 promoter, DNA polymorphism of resistant Vannamei was greater than that of WSSV susceptible Vannamei.

Group D was thought to have resistance to WSSV infection naturally. It was indicated by the presence of a consistent DNA band before and after the challenge test. Group D showed a specific Vannamei marker measuring 1650 bp. However, the livelihood of group D was low due to maintenance factors.

The 1650 bp band was found in all live shrimp after WSSV infection, including groups B and C. Thus, the 1650 bp DNA fragment had the opportunity to be a marker for Vanname shrimp resistant to the WSSV virus. Differences in DNA sequences between individuals in the oligonucleotides' primary binding region cause differences (polymorphisms) in the array pattern resulting from the amplification process. It reflects genetic diversity (Rafalski et al 1991).

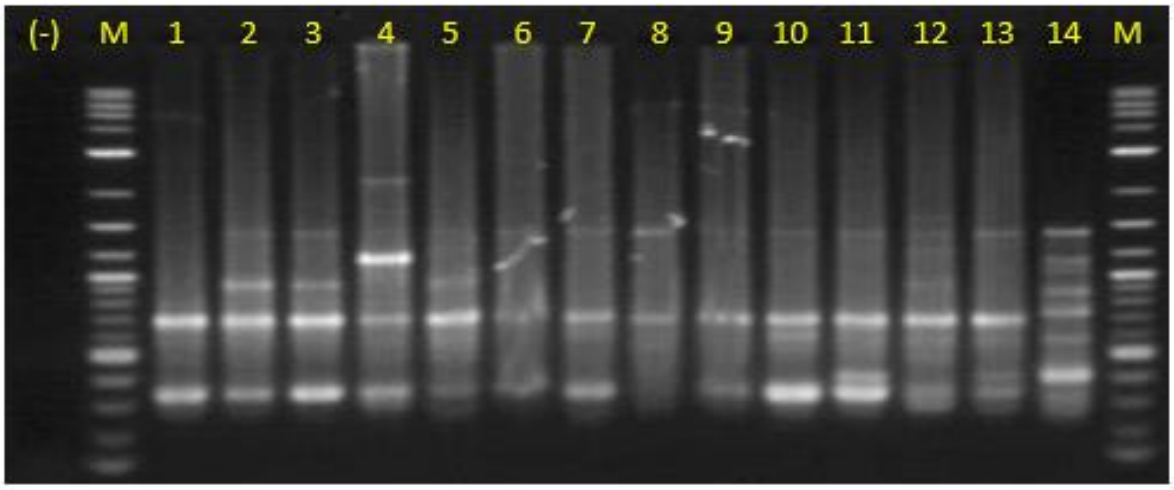

Figure 4. The DNA electrophoresis results of resistant/live and susceptible/dead Vanname shrimp PCR-RAPD products after the WSSV challenge test. The PCR process uses the OPD-02 primer. M: Marker 0.1-10 kilobase (kb), (-) negative control, lane (1-5) live WSSV infection in groups A, B, C, D, lane (6-9) dead WSSV infection in group A , B, C, D, lane (10-13) without WSSV infection in groups A, B, C, D, row (14) isolates WSSV (10^{-1}) LD50

The amplification results using OPD-02 primers showed that the specific DNA bands of Vanname shrimp markers had three ribbons with a size of 300, 700, and 1200 bp. The three DNA bands were owned by all live shrimp from control and treatment and WSSV susceptible shrimp. DNA fragments with sizes 300, 700, and 1200 bp were specific markers for Vanname. In the use of OPD-02 primer, the number of resistant Vanname shrimp DNA banding patterns ranged from 3-5, while susceptible Vanname shrimp had 3 DNA bands.

The WSSV-resistant Vanname shrimp group had a specific DNA fragment with a length of 1kb, namely 60% (3 samples in lanes No.2, 3, 5) and 1.1 and 1.4kb, namely 20% (1 sample in column 4). In contrast, the WSSV susceptible Vanname shrimp group does not have specific DNA fragments. This indicates that the DNA polymorphism of resistant Vanname shrimp is greater than that of sensitive Vanname shrimp. Thus, the DNA fragment of 1kb in length has the opportunity to become a marker for Vannamei resistance to the WSSV virus. The resistant Vannamei had the chance to appear marker 1kb more often (3 times) than DNA bands of size 1.1 and 1.4kb in WSSV resistant Vanname shrimp (only once).

The number of polymorphic fragments that appeared using OPC-09 primer was four fragments on WSSV resistant Vanname shrimp, and two monomorphic components were measuring 0.2 and 0.5 kb. The percentage of polymorphism in Vanname was resistant to WSSV 66.7%. For OPD-02 primers, three polymorphic fragments were obtained on WSSV resistant Vanname, while the number of monomorphic fragments was 3, 0.3, 0.7, and 1.2 kb. The number of monomorphic and polymorphic fragments of each WSSV-resistant and WSSV-susceptible Vannamei population is shown in Table 2.

Table 2. The number of monomorphic, polymorphic fragments, total fragments, percentage of polymorphisms, and fragment size ranges of WSSV-resistant and WSSV-susceptible Vanname shrimp populations.

Primer	the number of monomorphic fragment			the number of polymorphic fragment			Fragment total			Percentage of polymorphisms (%)			fragment average
	K	H	R	K	H	R	K	H	R	K	H	R	
OPC-09	2	2	2	3	4	3	5	6	5	60	66,7	60	0,2-4kb
OPD-02	3	3	3	0	3	0	3	6	3	0	50	0	0,3-4kb

Description: K: Vanname shrimp live without WSSV infection
H: Vanname shrimp live after WSSV infection
R: Vanname shrimp died after WSSV infection

The amplification results with OPD-02 primer showed a relatively high percentage of polymorphism, namely 50%. According to Warr (2003), the greater the diversity of an individual character or population, the better (profitable) it is. In table 2, the total number of fragments was produced from the two primers. Moreover, it shows the variation in the number of Vanname fragments that were resistant to WSSV. Specific markers were obtained by using particular primers to determine shrimp disease resistance. It could be used in superior shrimp production programs.

Table 3. The range of maintaining water quality in each treatment.

Parameter	Group			
	A	B	C	D
Salinity	30-31	30-32	30-31,5	29-31
pH	6,9-7,5	7,1-7,8	7,0-7,5	6,5-7,2
Ammonia	<0,1 ppm	<0,1 ppm	<0,1 ppm	<0,1 ppm
Nitrite	<0,01 ppm	<0,01 ppm	<0,01 ppm	<0,01 ppm

The average water quality obtained during the study was generally still in the acceptable range for Vanname shrimp's survival. The piping and replacement of water carried out can maintain the stability of the quality of maintenance water. The maintenance water quality table is shown in table 3.

4. Conclusion

Vanname DNA fragment profiles using OPC-09 primers showed 2 DNA bands specific to Vanname markers with sizes 200 and 500 bp. The average number of DNA bands resistant to 3 fragments, while Vannamei was susceptible to 1-2 fragments. The DNA polymorphisms of WSSV resistant Vanname were more significant than sensitive to WSSV. The 1650 bp band was found in all WSSV resistant shrimp groups. Thus, the 1650 bp DNA fragment had the opportunity to become a marker for Vannamei resistance to the WSSV virus.

Moreover, Vannamei DNA fragment profiles using OPD-02 primers showed that the specific DNA bands of Vanname shrimp markers were three ribbons with a size of 300, 700 and 1200 bp. The WSSV-resistant Vannamei group had a particular DNA fragment of 1kb in length, while the WSSV-susceptible group had no specific DNA fragment. The specific DNA fragment 1kb in size had the opportunity to become a marker for Vanname shrimp resistant to the WSSV virus.

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